

genomic sequence is in a database, the most efficient identification method presently available is matrix-associated laser desorption ionization mass spectrometry (MALDI-MS) with which about 500 spots can be analysed daily by one person. The method tolerates small amounts of salt in the sample, so that no time-consuming desalting steps are required after digestion. Several approaches using a combination of protein digestion on membranes and MS have also been reported. **Table 2** summarizes the essential steps of 2D electrophoresis and protein analysis.

Future Developments

2D electrophoresis is still in a developmental stage. Several technical improvements, mainly concerning further simplification of the technology and possible automation, an increase in reproducibility and sensitivity, and expansion of the pH detection spectrum, have to be achieved in order for the method to become routine in any biochemical laboratory. Gel grinding techniques, together with sophisticated software using the mass spectroscopic data, may be developed to produce a gel image without previous staining of the gel. Such a development could be decisive as to whether the technology will reach its major goal, i.e. the investigation of biological problems by a faithful comparison of protein expression levels. The completion of the sequencing of more genomes together with improvements in the analytical techniques will also

lead to a more widespread application of the technology.

See Colour Plate 43.

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Two-dimensional Polyacrylamide Gel Electrophoresis

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Introduction

The evolution of tools utilized in biology and medicine, together with the exponential progress accomplished recently in the area of bioinformation, enables analysis of whole organism constituents. Such analyses are best exemplified by complete genomic sequences of different microorganisms, and by the recent development in techniques permitting dissection of the whole protein repertoire of an individual, namely its proteome. Furthermore, the dramatic growth in the number of genome projects as

well as the speed with which genome sequences are determined has generated huge amounts of information. This progress has boosted techniques, notably two-dimension polyacrylamide gel electrophoresis (2D-PAGE), enabling the analysis of a proteome consisting of all the proteins expressed by a genome. Such analyses give information on the effector molecule itself, namely the protein, and take into account highly sophisticated mechanisms regulating gene expression. 2D-PAGE is the most powerful tool to separate a multitude of polypeptides that are contained in a single biological sample. Various procedures have been described to separate proteins according to biophysical parameters. In 1975, O'Farrel, Klose and Scheele described optimized 2D procedures in which proteins were denatured and separated by electrophoresis on polyacrylamide gel. The first gel dimension comprised a separation according to the protein charge by isoelectric focusing,

and the second gel dimension separated proteins according to their sizes. Thus, peptides are separated from one another according to two independent biochemical properties. 2D-PAGE was shown to be particularly valuable in the study, as well as in the identification of thousands of cellular or secreted proteins, including many of those present in human plasma/serum (Figure 1).

During the past few years, tremendous progress has been made in the field of 2D-PAGE. The 2D technique has been simplified, and, more importantly, made reproducible. Commercially manufactured im-

mobilized pH gradients (IPGs), with both acidic and basic high resolution power and precast sodium dodecyl sulfate (SDS) PAGE gels are now available. In addition, progress in protein solubilization and in the development of systems allowing high loading capacities has been made.

More than 20 years after its birth, 2D-PAGE is now a major technique in protein sciences. Over the past few years there has been an exponential increase in the creation and expansion of protein databases such as the SWISS-2DPAGE, the HEART-2DPAGE and the HSC-2DPAGE. Furthermore, tools have been developed to

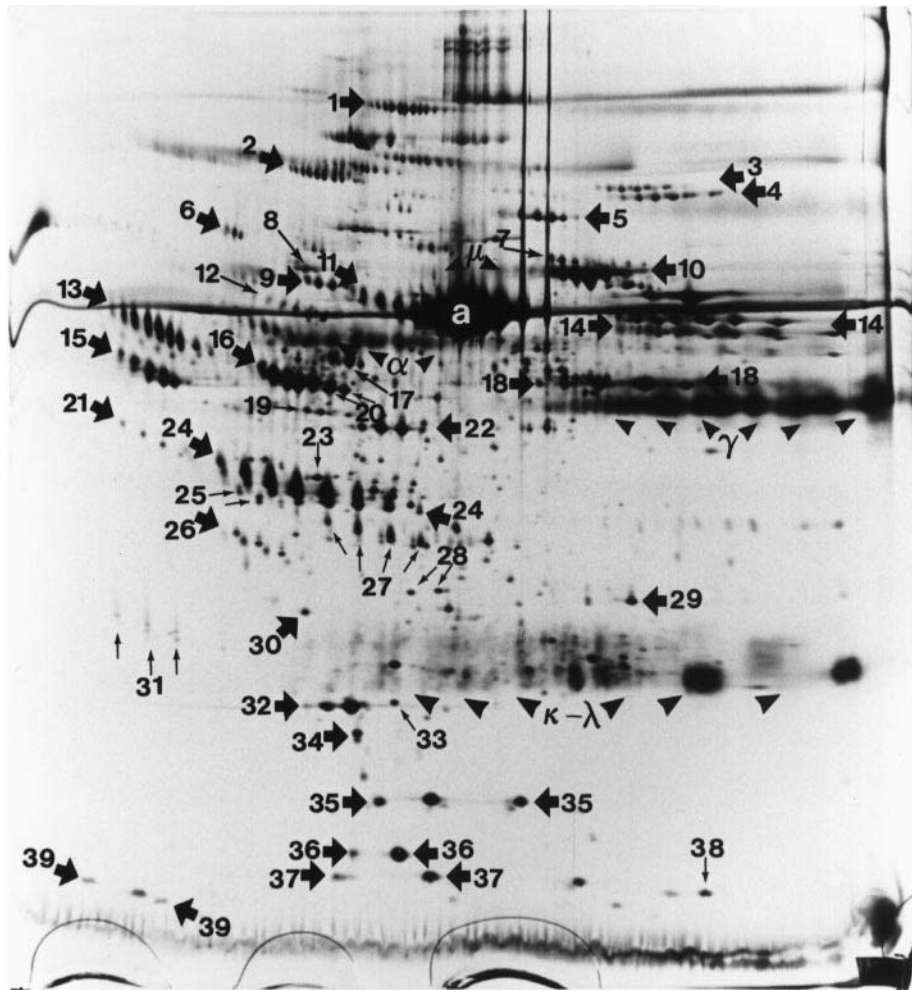


Figure 1 The normal human plasma 2D map. Polypeptides (0.3 μ L of plasma) were separated by pH 3.5–10 carrier ampholyte gradient, followed by gradient 9–16% T polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The ammoniacal silver-stained gel was photographed with the higher molecular weight at the top and the acidic side on the left. 1, α_2 -macroglobulin; 2, ceruloplasmin; 3, glu-plasminogen; 4, lys-plasminogen; 5, complement factor B; 6, complement C1s; 7, protransferrin; 8, prothrombin; 9, α_1 -B-glycoprotein; 10, transferrin; 11, hemopexin; 12, α_2 -antiplasmin; 13, α_1 -antichymotrypsin; 14, fibrinogen α chain; 15, α_2 -HS-glycoprotein; 16, α_1 -antitrypsin; 17, antithrombin III; 18, fibrinogen β chain; 19, extended fibrinogen γ chain; 20, Ge-globulin; 21, lysin-rich glycoprotein; 22, fibrinogen γ chain; 23, apolipoprotein A-IV; 24, haptoglobin β chain; 25, Zn- α -glycoprotein; 26, apolipoprotein J; 27, cleaved haptoglobin β chain; 28, apolipoprotein E (phenotype E 3/4); 29, γ chain of complement C₄; 30, α_1 -microglobulin; 31, apolipoprotein D; 32, apo A-I; 33, proapolipoprotein A-I; 34, retinol-binding protein; 35, haptoglobin α_1 chain; 36, transthyretin (prealbumin); 37, haptoglobin α_2 chain; 38, haemoglobin β chain; 39, apolipoproteins C-II and C-III; a, albumin; μ , polyclonal heavy chains of IgM; α , polyclonal heavy chains of IgA; γ , polyclonal heavy chains of IgG; κ - λ , polyclonal Ig light chains. Reproduced with permission from Tissot and Spertini (1995).

compare 2D gels images across the Internet. Most importantly, methods for the analysis of 2D gels are continually improving. The high sensitivity and throughput of these techniques now enable the characterization of hundreds of proteins from a whole cell, tissue or body fluid. Proteins can be identified according to primary parameters such as their isoelectric points, apparent molecular mass, real mass and protein N- or C-terminal sequence tag, but also according to secondary attributes such as peptide mass fingerprint, peptide fragmentation data or amino acid composition. Interfacing and integrating databases from 2D gels such as SWISS-2DPAGE, SWISS-PROT, GenBank, EMBL nucleotide sequence database, dbest, GSBD and the NLM's MEDLINE bibliographical reference database provide researchers with invaluable tools to study both genome and proteome. The continuing progress accomplished in both proteome research and bio-information will contribute to the implement of the *Cyber-Encyclopedia of the Proteome*, as suggested by R.D. Appel. This development increases the need for simple protocols to run reproducible 2D gels and is an important step for investigators involved in proteomics. Several well-written protocols and reviews are accessible in the literature or through the World Wide Web (<http://expasy.hcuge.ch/ch2d/technical-info.html>; <http://www.abdn.ac.uk/~mmb023/2dhome.htm>). We will review here some of the important features that must be evaluated in order to implement a new 2D-gel laboratory.

Sample Preparation and Protein Solubilization

2D-PAGE is an ideal separation tool. Nevertheless, sample preparation and protein solubilization are still a key step that is frequently ignored. In addition, there is no universal and ideal sample buffer for 2D-PAGE. The goal of sample preparation is to maximize solubilization and disaggregation of the tissues while preventing protein degradation. For these reasons, and because samples have various characteristics, protocols have to be adapted according to the origin of the samples. Sample preparation is quite easy with soluble proteins such as those of plasma or cerebrospinal fluid, but presents major difficulties in the presence of membrane or nuclear proteins. Tissue samples are usually mechanically disrupted (ultrasonication, rapid agitation in the presence of glass or zirconium microspheres), washed in a low salt buffer and then chemically (lysis buffer) solubilized. Constituents such as nucleic acids, lipids or salts can interfere with both solubilization and with the electrophoretic properties of the proteins. Before loading, nonsolubilized material must be eliminated by high

speed centrifugation. To break down interpolypeptide disulfide bonds and to maintain reducing conditions, components such as dithiothreitol, dithioerythritol or β -mercaptoethanol are used. The solubility problem can be alleviated to a large extent by the proper use of a combination of chaotropes and detergents in combination. Urea is a common constituent of protein sample preparations, and in its presence care must be taken to avoid heating above 32°C, which causes carbamylation of peptides. Impressive improvements in protein solubilization have been obtained with a denaturing solution containing urea, thiourea and detergents (both nonionic and zwitterionic). The ideal conditions would combine the highest chaotropic power (i.e. 2 mol L⁻¹ thiourea and 7–8 mol L⁻¹ urea) with a detergent cocktail (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, Triton X-100).

The main problem encountered with thiourea is the strong inhibition of acrylamide polymerization. Another problem complicating protein separation by 2D-PAGE is that, at high concentrations, many proteins are prone to precipitation, resulting in poor resolution after isoelectric focusing. In order to load large amounts of proteins, investigators have overcome the problem by using 'in gel' application of the samples, avoiding the use of sample cups and eliminating precipitation at the same application site.

Isoelectric Focusing

In 1964, synthetic carrier ampholytes (aliphatic oligoamino and oligocarboxylic acids) were synthesized allowing separation of peptides according to their charges. For 2D-PAGE, carrier ampholyte isoelectric focusing is usually performed in an ampholyte–polyacrylamide matrix that is polymerized in a glass tube. After sample loading, polypeptides are concentrated into narrow bands within a continuous pH gradient in the polyacrylamide gel matrix. Proteins migrate in an electric field until they arrive at a position in which they have no net charge, i.e. their isoelectric point (pI). Isoelectric focusing is useful because: (i) no diffusion of proteins occurs because of the focusing effect; (ii) it offers a resolution allowing the separation of microheterogeneous populations of proteins; and (iii) the pI of the protein can be estimated. In the past, pH gradients were generated by carrier ampholytes or amphoteric buffer moving freely within an acrylamide matrix.

Practically, many factors can affect measurement of the apparent pI of the proteins during isoelectric focusing with carrier ampholytes: (i) since the proteins have their own inherent charges, they can act as ampholytes themselves and affect pH during focus-

ing; (ii) the carrier ampholytes have a higher mobility than the proteins; (iii) some proteins may never reach steady state due to polyacrylamide gel matrix restriction; (iv) ampholyte-protein interactions may alter the observed *pI* of the proteins. Temperature, time, voltage and salt concentration are other parameters that may dramatically influence the determination of the *pI*. Finally, the basic proteins are not detected without using nonequilibrium pH gradient electrophoresis. Nowadays, many of these problems have been resolved by the development of isoelectric focusing with an IPG. The pH gradient is created by copolymerization of acrylamide/*N,N'*-methylenebisacrylamide with acrylamido derivatives, containing either carboxyl or tertiary amino groups as buffers and sulfate groups (acidic) or quaternary ammonium (basic) as strong titrants (Immobilines). This method is a true equilibrium method, which significantly improves the feasibility of the 2D-PAGE.

Recently, highly reproducible, commercially available, wide-range as well as narrow-range IPGs have been produced. The latter gradients allow a pH scale that enables comparison of several 2D gel maps generated with many IPGs in the first dimension and with various biological samples. IPGs also offer the possibility of determining *pI* without major differences from the calculated *pI* values, unless there are significant post-translational protein modifications. Nowadays, with the improvements of IPG production, it is possible to detect proteins with *pIs* up to pH 12 in a single IPG gel with highly reproducible protein patterns.

Finally, as mentioned in the previous section, entire IPG gels can be used for sample application, with the protein entering the gels during their rehydration. This approach is useful because it eliminates precipitation at the sample application site, it improves the resolution over the entire pH range of the gels, and it allows precise control of protein amounts and sample volumes loaded on to the IPG gels. Up to 5 mg of proteins can be loaded on wide IPG gels and up to 15 mg on narrow pH range gels. Contrarily to isoelectric focusing with carrier ampholytes, electroendosmosis (transport of water towards the cathode at low pH values or towards the anode at high pH values) is generally not a problem with IPGs. Currently, isoelectric focusing using carrier ampholytes still has a place in a 2D laboratory, because the resolution of particular proteins is sometimes better.

From the First to the Second Dimension

Transfer of the first-dimension spaghetti-like gels after isoelectric focusing in the presence of am-

pholytes used to be a manipulation challenge for beginners in the field of 2D-PAGE. Extrusion of the gels from the glass tubes was difficult, and the gels frequently broke into several pieces. With the use of IPG strips, which are deposited on a plastic backing material, the transfer is easy. Practically, after the first dimension run, the strips are equilibrated in buffers containing SDS in order to maintain proteins in solution and to reduce -S-S- bonds. Subsequently, strips are placed over SDS gels that may or may not contain a stacking gel.

SDS-PAGE

In the second dimension, polypeptides are separated according to their size in a gel matrix. Practically, after electric focusing in reducing conditions, proteins are separated into their polypeptide components. The latter are mixed with SDS-containing buffers. SDS binds to polypeptides at a constant mass ratio (1.4 g SDS per gram of protein). As a consequence, polypeptides organize as rod-like molecules, with a diameter of 1.8 nm, and their lengths depend on their molecular weight. The bound SDS molecules contribute to a strong negative charge, which effectively swamps the intrinsic charge of the polypeptides. Thus, in general the SDS-polypeptide complexes have the same mass/charge ratio and, in a sieving polyacrylamide matrix, they will migrate according to their molecular weight. Glycoproteins and lipoproteins can migrate abnormally as they are not easily saturated with SDS.

The gel matrix is most frequently composed of polyacrylamide generated by polymerization of acrylamide monomers into long chains that are cross-linked. Usually, cross-linkers are bifunctional components such as *N,N'*-methylenebisacrylamide (bis) or diacrylpiperazine. Polymerization of acrylamide is initiated either by the use of ammonium persulfate or riboflavin, and is accelerated by the use of *N,N,N',N'*-tetramethylethylenediamine (TEMED). Oxygen inhibits polymerization, and thus gel mixtures are usually degassed. The composition of a polyacrylamide gel is defined by two parameters: % *T* and % *C*. The % *T* (w/v) is the total concentration of the monomer (acrylamide plus cross-linker), whereas % *C* corresponds to the ratio (w/w) of the cross-linker to the acrylamide. The pore size of the polymerized acrylamide will depend on these two parameters, but since pore formation is random, pore sizes will never be totally uniform. The choice of the mean pore size will depend on the molecular weight of the proteins to be studied. The second-dimension SDS-PAGE can be performed with home-made vertical or horizontal systems, using linear or gradient polyacrylamide

(9–16%) gels. Commercially manufactured gels are also available. The advantages of the latter reside in their reproducibility and safety (polymerized acrylamide being clearly less neurotoxic as compared with monomeric acrylamide). The sensitivity as well as the resolution power of the protein detection must be kept in mind before choosing optimal conditions for SDS-PAGE. Gels polymerized with the photoinitiator system, composed of methylene blue, toluene sulfinate and diphenyliodonium chloride, lead to low resolution power after silver staining. Resolution can be restored if methylene blue is replaced by riboflavin. Gels polymerized with the riboflavin/sulfinate/iodonium system yield better results upon *N*-terminal microsequencing after blotting than gels polymerized with the standard TEMED/ammonium persulfate system.

Protein Visualization

Several methods have been described to detect protein spots after 2D-PAGE. These methods use reactants

such as Coomassie Brilliant Blue R-250, Amido Black, Ponceau S, Fast Green, negative staining, silver staining, fluorescein and radioisotopes. The two most popular approaches are Coomassie Brilliant Blue R-250 and silver staining. A good stoichiometric relationship has been documented between protein abundance and integrated optical density of protein spots for Coomassie Brilliant Blue R-250. The silver staining methods are more sensitive than those using Coomassie Blue and can detect as little as 1–4 ng of proteins. Several methods of silver staining of proteins have been described, with the most rapid ones being usually less sensitive and less reproducible than the more time-consuming ones. Among the latter methods, those using silver–diamine complex give the most uniform sensitivity. However, they require special home-made gels and cannot be applied to several electrophoretic systems. For these reasons, protocols based on silver nitrate are of more general use and are favoured. A variety of systems using different metal cations (K^+ , Cu^{2+} , Zn^{2+}) has been

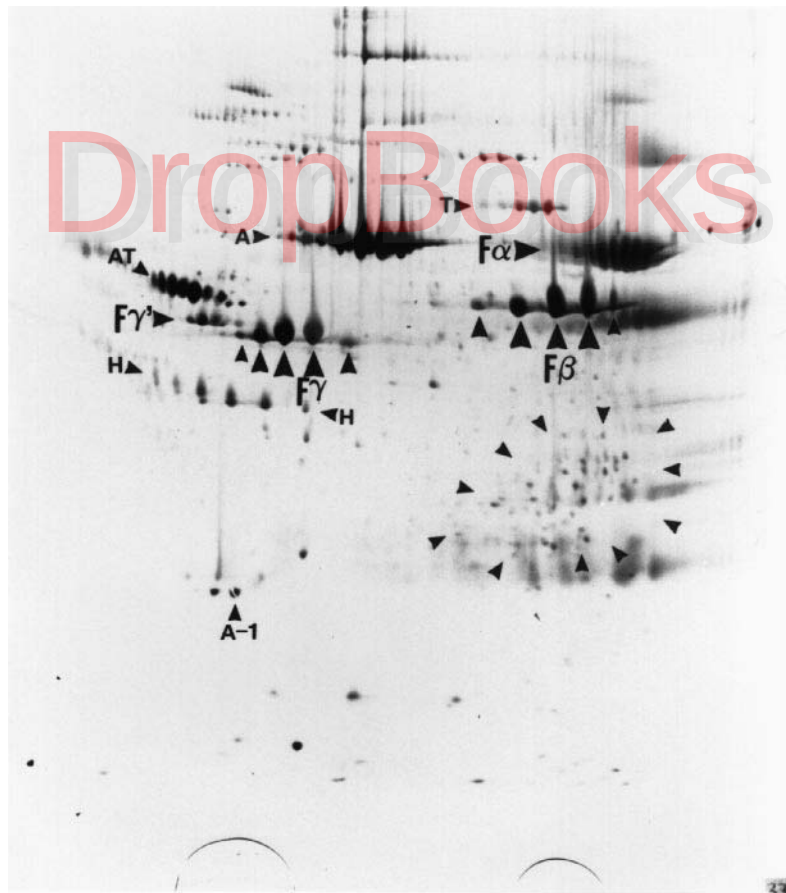


Figure 2 Microheterogeneity of proteins. 2D gel of a cryoprecipitate containing fibrinogen (cryofibrinogen). A, albumin; AT, α_1 -antitrypsin; T, transferrin; H, haptoglobin β chain; A-1, apolipoprotein A-1; $F\alpha$, fibrinogen α chain; $F\beta$, fibrinogen β chain; $F\gamma$, fibrinogen γ chain; $F\gamma'$, extend fibrinogen γ chain. Unknown protein spots are shown by arrowheads. All major identified proteins present charge microheterogeneities. First dimension: immobilized pH gradient.

developed to stain SDS-PAGE separated proteins without the need for fixative, organic dye or chemical modifier. SDS proteins stain negatively upon gel treatment with solutions of heavy metal salts. The zinc imidazolate reverse-staining method offers the advantage of combining good sensitivity, rapidity and reversible interaction. Furthermore, the zinc imidazolate reverse-staining method can be used in situations where Coomassie Brilliant Blue R-250 or silver staining is inappropriate or fail to produce detection of the polypeptides of interest.

Protein Microheterogeneity

Polypeptides separated by 2D-PAGE rarely appear as single spots, and most are resolved as multiple spots characterized by charge and size microheterogeneities (Figure 2).

Microheterogeneity is due to several factors that frequently occur together. The first cause of microheterogeneity is genetic polymorphism where heterozygote individuals express both forms of the gene (Figure 3); the second is related to protein co- and post-translational modifications. These modifications are multiple and have all the potential of modifying a protein's charge, hydrophobicity, conformation and/or stability. Furthermore, the 'one gene one polypeptide' paradigm is challenged by the alternative splicing of many genes responsible for synthesizing several proteins from a single gene. An important feature of 2D gel analysis is that various protein isoforms generated by co- and/or post-translational modifications can be separated by isoelectric focusing and/or by SDS-PAGE. Among the modifications which lead to a charge-dependent change to a protein, acylation, alkylation, carboxymethylation, phosphorylation, sulfation, carboxylation, sialylation and proteolytic processing are involved. Finally, glycosylation of proteins may lead to both charge and size modifications, and microheterogeneity of a protein may reflect the presence of several glycoforms.

Protein Identification

Several approaches have been used to identify proteins after 2D-PAGE. Co-migration with purified known proteins and Western blotting were employed by the pioneers of the 2D field. The use of specific antibodies and the recent developments of antigen-antibody interactions with enhanced chemiluminescence allow detection and identification of traces of proteins. However, monoclonal antibodies may not detect denatured polypeptides. Nowadays, proteins are identified by microsequencing, amino

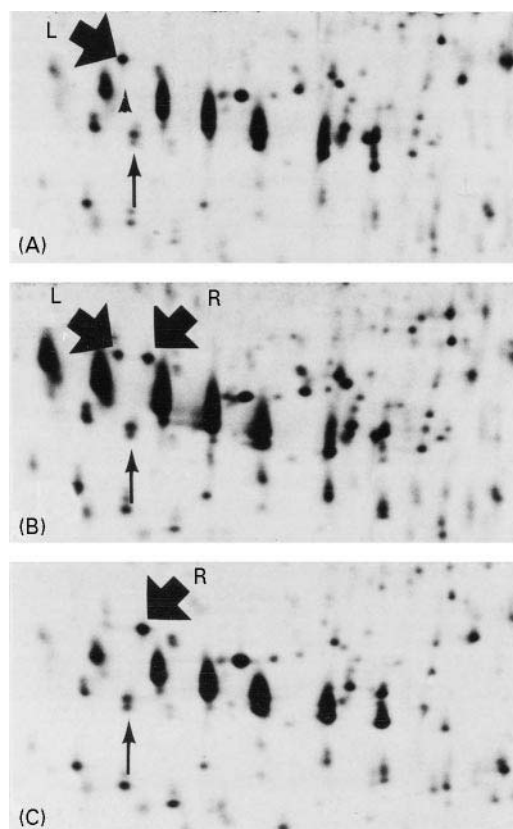


Figure 3 Genetic heterogeneity of plasma proteins. Close-up of the human plasma 2D map corresponding to the haptoglobin β chain (pH 3.5–10 carrier ampholyte gradient). (A) L form (homozygous); (B) L–R forms (heterozygous); (C) R form (homozygous). A reference spot is shown by the arrowhead. This genetic polymorphism is less detectable with immobilized pH gradients. Note the microheterogeneity of haptoglobin β chain spots, which present both charge and size heterogeneities.

acid analysis, peptide mass fingerprinting and/or mass spectrometry. The development of automated, high throughput technologies for the rapid identification of proteins is in progress. Automation already exists in several stages of the protein identification process. This includes automated acquisition of matrix-assisted laser desorption ionization-time-of-flight mass spectra and peptide mass fingerprinting. Bioinformation allows identification of proteins by mixing several databases (a classified index can be found at the following address: <http://expasy.hcuge.ch/alinks.html#Proteins>).

Data Management

Many investigators have analysed their 2D gels by holding two, sometimes three gels together towards a light source, and tried to identify differences between them (Figure 4). However, analysis of a multitude of 2D gels, with its bulk of information is greatly

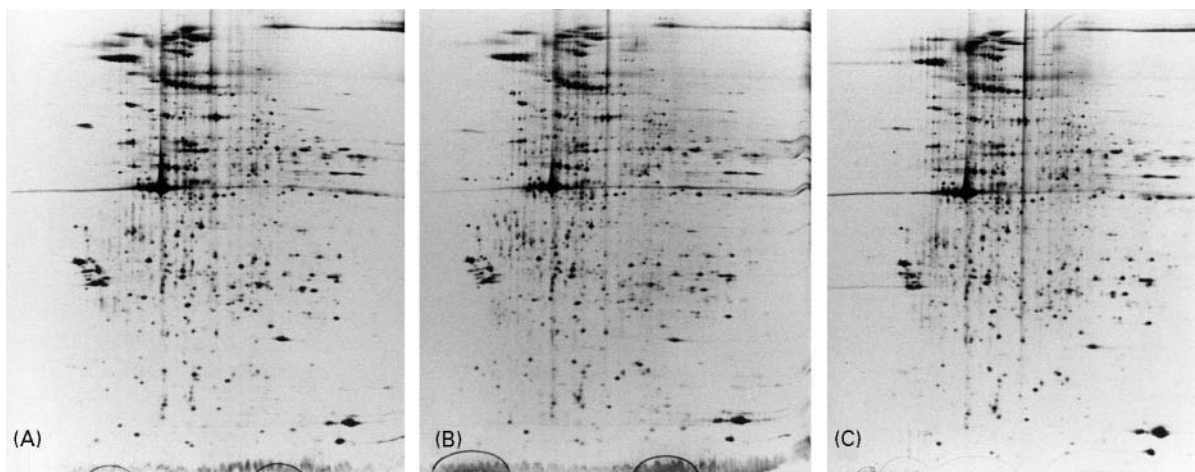


Figure 4 Analysis of 2D gels. 2D gels of human platelets from a single blood donor (pH 3.5–10 carrier ampholyte gradient). (A) Platelets stored with leukocytes, 1 day after collection; (B) platelets stored in the presence of leukocytes, 7 days after collection; (C) platelets stored in the absence of leukocytes, 7 days after collection. Without computerized analysis, it is not possible to draw a definitive conclusion from the comparison of this set of gels. Reproduced with permission from Sarraj-Reguieg A, Tissot JD, Hochstrasser DF, von Fliedner V, Bachmann F and Schneider P (1993) Effect of prestorage leukocyte reduction on proteins of platelets obtained by apheresis. *Vox Sanguinis* 65: 279.

facilitated by the use of computer-based data processing. The improvements in image acquisition and image analysis allow clear spot detection, background subtraction, spot matching and database construction. Furthermore, interpretation of 2D-PAGE images is facilitated by statistical methods, artificial intelligence and machine-learning programs. Ascendant hierarchical classification sorts the image into meaningful groups. The use of correspondence analysis and ascendant hierarchical clustering allows identification of new, potentially important proteins. Many database of 2D gel master images are accessible through the World Wide Web (Internet sites can be found at the following addresses: <http://www.expasy.ch/ch2d/2d-index.html> or <http://www-lmmb.ncifcrf.gov/ABRF97/abrf3.html>). It is also possible to compare 2D gels from various laboratories, or 2D gels with masters, on the World Wide Web by using the flicker created by P.F. Lemkin (accessible at <http://www-lecb.ncifcrf.gov/flicker/>).

Protein Functions

After 2D-PAGE analysis of cells, thousands of spots are observed. Such an observation is frequently impressive, but not very useful. Identification of the polypeptide sequence corresponding to a spot is already undergoing major progress. However, understanding the protein's function(s) remains the final goal of any analysis. It is also relevant to study the expression level, the phosphorylation state, the sub-cellular location, the association with other proteins and the rate of synthesis or degradation of the

proteins of interest. Combination of all this information will make possible the study of a functional proteome.

Concluding Remarks

Amino acids are like letters. Amino acids make proteins; letters make words. Proteins are like words. Some are known, others are unknown. Proteome databases are like dictionaries; they contain a lot of information and are very useful. Organization of the words makes the texts; organization and regulation of protein production make the cells. 2D-PAGE is a major proteomics tool. The technique should be applied to resolve specific biological problems. It should not be used for random investigations.

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EXTRACTION



Analytical Extractions

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Introduction

The process of generating analytical data involves some combination of planning, sampling, sample preparation, quantification, data review and reporting. Initially, each step in the method required comparable effort. Sample preparation, generally involving some form of extraction followed by analyte enrichment, has in the past been a laborious process, with only a few tools available. Likewise, quantification usually consisted of a 'wet' chemistry process such as titration or precipitation. Before the development of personal computers, the planning, sampling, data review and reporting steps also required considerable effort. Since each step presented formidable challenges to the analytical scientist, the relative importance of each step remained about the same.

Modern techniques, particularly chromatography, have changed the situation. The rapid and successful development of gas and liquid chromatography dramatically reduced quantification steps from hours or days to a matter of minutes, often with better accuracy and precision. The other steps in the method,

especially sample preparation, were regarded as of secondary importance, serving only to support the ultimate (i.e. chromatographic) step in the method. Since most of the creative – and financial – resources flowed into chromatography development, research in the other areas slowed and sample preparation came to be viewed as the 'low tech' part of the method.

Chromatography is now considered a mature science, being an integral part of nearly every analytical laboratory. The slower pace of chromatographic research, coupled with outside pressures to improve the efficiency of the entire analytical method, has finally resulted in an increased interest in sample preparation. These efforts have produced a number of advances that improve efficiency, selectivity and time required. The discussion will provide an overview of some of the many sample preparation principles and techniques available, focusing on the analytical extraction part of the process. The goal is to provide the reader with a more balanced view of this important part of analytical methodology.

Principles of Extraction

Developing a successful extraction as part of an analytical method requires an understanding of the chemical and physical principles involved. Thus, we will begin this discussion of analytical extraction by focusing on the underlying principles that make the techniques work. Only with understanding and